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Modulation of Bile Acid Metabolism by 1α-Hydroxyvitamin D_3 Administration in Mice

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Running title: Modulation of bile acid metabolism by vitamin D_3

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid;

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LCA, lithocholic acid; FXR, farnesoid X receptor; PXR, pregnane X receptor; VDR, vitamin D receptor; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; Cyp, cytochrome P450; Asbt, apical sodium-dependent bile acid transporter; MRP, multidrug resistance-associated protein; $1\alpha(OH)D_3$, 1α-hydroxyvitamin D_3; HDCA, hyodeoxycholic acid; UDCA, ursodeoxycholic acid; MCA, muricholic acid; GC-MS, gas chromatography-mass spectrometry; Ntcp, sodium taurocholate-cotransporting polypeptide; Oatp, organic anion transporting polypeptides; Bsep, bile salt export pump; Ost, organic solute transporter.

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Abstract

The vitamin D receptor (VDR) is a nuclear receptor for the active form of vitamin D_3 and mediates regulation of calcium homeostasis. Bile acids, such as lithocholic acid, have been identified as additional endogenous VDR ligands. The *in vivo* role of VDR in bile acid metabolism has not been elucidated. We investigated potential effects of *in vivo* VDR activation on bile acid metabolism by feeding mice bile acid-supplemented chow and then treating with 1α-hydroxyvitamin D_3 [1α(OH)D_3]. We administered 1α(OH)D_3 via gavage to mice fed chow supplemented with 0.4% cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), or lithocholic acid (LCA) and examined liver and plasma bile acid composition with gas chromatography-mass spectrometry analysis. 1α(OH)D_3 treatment reduced hepatic bile acids in mice fed CDCA- and DCA-supplemented chow, but was less effective in mice fed chow supplemented with LCA or CA. 1α(OH)D_3 administration also decreased plasma bile acids in mice fed bile acids, such as DCA. The effect of 1α(OH)D_3 administration in decreasing liver bile acid composition was observed in mice under fasting conditions and was associated with increased urinary excretion and increased expression of bile acid

transporters, such as renal multidrug resistance-associated protein 4. These findings indicate that pharmacological activation of VDR enhances metabolism of bile acids, especially urinary excretion. The results confirm that VDR acts a regulator of bile acid metabolism *in vivo*.

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Introduction

Bile acids are essential detergents required for the digestion and intestinal absorption of hydrophobic nutrients, such as fatty acids, cholesterol and lipid-soluble vitamins, including vitamin D (Hofmann, 1999). Bile acids are the major products of cholesterol metabolism and play an important role in the elimination of cholesterol by inducing biliary lipid secretion and the solubilization of cholesterol in bile. Primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are generated from cholesterol by the sequential actions of liver enzymes, and are secreted in bile as glycine or taurine conjugates (Russell, 2003). After assisting in lipid digestion and absorption, most bile acids are reabsorbed in the intestine and recirculate to the liver through a mechanism called the enterohepatic circulation. Bile acids that escape reabsorption are converted to secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA), by the intestinal microflora (Ridlon et al., 2006). Bile acids are cytotoxic at elevated concentrations, and secondary bile acids are considered to be involved in the pathogenesis of gallstone disease and colon cancer.

Bile acid metabolism is regulated at several levels, including gene transcription,

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RNA translation and protein stability (Russell, 2003). Bile acids act as steroid hormone-like regulatory signals for nuclear receptors, which regulate the expression of genes involved in bile acid synthesis and transport (Makishima, 2005). The farnesoid X receptor (FXR; NR1H4) binds to primary and secondary bile acids, represses bile acid synthesis and hepatocellular import, stimulates bile acid export from cells, and protects hepatocytes from bile acid toxicity. The pregnane X receptor (PXR; NR1I2) senses toxic secondary bile acids and induces their elimination through a xenobiotic metabolism pathway. The vitamin D receptor (VDR; NR1I1), a receptor for 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, calcitriol], also acts as a bile acid receptor with specificity for the secondary bile acid LCA and its derivatives (Makishima et al., 2002). While the roles of VDR in calcium and bone homeostasis have been investigated for decades, an understanding of the biology of VDR regulation of bile acid metabolism is only now emerging. VDR induces the intestinal expression of mouse cytochrome P450 3a11 (Cyp3a11) and human CYP3A4 (Thummel et al., 2001; Matsubara et al., 2008). CYP3A enzymes are predominantly expressed in the liver and intestinal mucosa and catalyze the metabolic conversion of a wide diversity of xenobiotics and endogenous substrates,

including bile acids, to more polar derivatives (Xie and Evans, 2001). Dehydroepiandrosterone sulfotransferase 2A1, apical sodium-dependent bile acid transporter (Asbt) and multidrug resistance-associated protein 3 (MRP3) have been demonstrated to be VDR target genes in mouse and human cells (Echchgadda et al., 2004; McCarthy et al., 2005; Chen et al., 2006). Although these proteins are involved in bile acid metabolism, the *in vivo* role of VDR in bile acid metabolism has not been elucidated. In this study, we examined the effects of 1α-hydroxyvitamin D_3 [1α(OH)D_3, alfacalcidol] on bile acid composition in mice fed bile acid-supplemented chow and found that VDR activation stimulates metabolism of bile acids such as CDCA.

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Materials and Methods

Compounds. 1α(OH)D_3 was kindly provided by Dr. Yoji Tachibana (Nisshin Flour Milling Co., Saitama, Japan). CA, DCA, CDCA, LCA, hyodeoxycholic acid (HDCA) and ursodeoxycholic acid (UDCA) were purchased from Sigma-Aldrich (St. Louis, MO), and α-muricholic acid (α-MCA), β-MCA and ω-MCA were from Steraloids, Inc. (Newport, RI). [1-^{14}C]-Glycocholic acid (50 mCi/mmol) was purchased from GE Healthcare (Chalfont St. Giles, United Kingdom).

Animals and Treatment. C57BL/6J male mice (5-6 weeks of age; Tokyo Laboratory Animals Science Co., Tokyo, Japan) were housed under controlled temperature (23 ± 1°C), humidity (45-65%), and standard 12-hour light/12-hour dark cycle. Prior to feeding of chow supplemented with bile acid, mice were fed standard rodent chow (Lab. Animal Diet MF; Oriental Yeast Co., Tokyo, Japan). For bile acid supplementation, standard chow was finely powdered and mixed thoroughly with CA, CDCA, DCA, or LCA at 0.4% (w/w) composition. In initial experiments, mice were fed powdered standard or 0.4% bile acid-supplemented chow for 8 days (Fig. 1A). On days 6,

7 and 8, mice were orally administered 1α(OH)D_3 dissolved in corn oil (0.2 mL) at a dose of 2.5 nmol/mouse per day (n=5-8). At day 9, mice were anesthetized with ether and blood was collected by cardiac puncture with a heparinized syringe. Tissue samples were collected, frozen on dry ice immediately after removal and weighing, and then stored at -80°C. In the second experiment, mice were fed chow or 0.4% CDCA-supplemented chow from day 1 until day 5, fasted from day 6, and administered 2.5 nmol/mouse 1α(OH)D_3 via gavage on days 6 and 7 (Fig. 1B). Forty-eight-hour urine samples (on days 7-8) were collected in glass metabolic bowls. The experimental protocol adhered to the Guidelines for Animal Experiments of the Nihon University School of Medicine and was approved by the Ethics Review Committee for Animal Experimentation of Nihon University School of Medicine.

Extraction and Derivatization of Bile Acids. For extraction of bile acids, liver samples (about 0.4 g) were homogenized in 6 mL of 80% (v/v) ethanol with a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) on ice and centrifuged at 9,400 × g for 10 min. Precipitates were re-homogenized with 6 mL of 80%

(v/v) ethanol with a Sonifier (Branson, Danbury, CT) and then centrifuged. Extraction with ethanol was repeated three times and combined supernatant from liver homogenate was evaporated to dryness. [1-^{14}C]-Glycocholic acid was added to a liver sample and the extraction yield was measured by liquid scintillation counting. The recovery yield of [1-^{14}C]-glycocholic acid was 94% (mean from triplicate assay). Plasma bile acids were extracted by solid phase extraction using a C18 Bond Elute column (Varian, Inc., Palo Alto, CA) as reported previously (Setchell and Worthington, 1982). Plasma samples (0.4 mL) were mixed with 1 mL of 0.5 mol/L triethylammonium sulfate, pH 7.5, and heated at 65°C for 15 min to liberate the protein-bound bile acids. After chilling, the mixture was applied to a C18 column, rinsed briefly, eluted with 4 mL of ethanol, and evaporated to dryness. Using a C18 Bond Elute column, quantitative yield is successful attained for both non-polar, such as lithocholic acid, and polar bile acids, including conjugated bile acids (Setchell and Worthington, 1982). We estimated the recovery yield of plasma bile acids from a C18 Bond Elute column using [1-^{14}C]-glycocholic acid. Over 98% of [1-^{14}C]-glycocholic acid was obtained in the ethanol solution by liquid scintillation counting.

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Extracted liver and plasma bile acids were subjected to gas chromatography-mass spectrometry (GC-MS) after derivatization. After the addition of an internal standard, bile acids were treated with 0.6 mL of acetone/methanol/6M HCl (36:4:0.4 by volume) at 37°C for 14 hours to remove the conjugated sulfonyl group (Parmentier and Eyssen, 1975), and then with 15% (w/v) NaOH at 120°C for 2 hours in an autoclave to deconjugate the taurine or glycine moiety (Keller and Jahreis, 2004). Samples were then extracted with 2 mL of hexane twice to remove cholesterol, and then acidified to approximately pH 1 with 2M HCl. Deconjugated bile acids were extracted with 2 mL of diethyl ether three times and converted to methyl esters with trimethylsilyldiazomethane (GL Sciences, Inc. Japan, Tokyo, Japan) and to trimethylsilyl derivatives with *N,O*-bis(trimethylsilyl)trifluoroacetamide plus trimethylchlorosilane (Thermo Fisher Scientific Inc., Rockford, IL).

GC-MS Analysis of Bile Acids. We utilized a GC-MS analyzer, Shimadzu GC-MS QP5050A, equipped with an autoinjector AOC-20i and a data system, GCMS Solution (Shimadzu Corporation, Kyoto, Japan). Gas-chromatographic separation was

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carried out with a separation column of HP-5 (cross linked 5% Ph-Me silicon, 0.32 mm internal diameter, 0.15 μm film thickness and 25 m in length; Agilent Technologies, Inc., Santa Clara CA). The injection temperature was 150°C and the column temperature was programmed at 150°C for 5 minutes, 10°C /minute to 260°C and held for 30 minutes. An ionizing energy was set at 70 eV, an ionizing trap current at 60 μA, and an ion detector gain at 1.1 kV. Highly purified helium gas was used as carrier gas at a flow rate of 1.0 ml/minute. Split ratio was 1:10, and sampling rate was 0.5 seconds. The bile acids were analyzed as methylester-trimethylsilyl derivatives (Setchell et al., 1983). An appropriate fragment ion in the high mass region was selected for mass fragmentography. Their fragment ions (m/z) and relative intensities (%) were as follows: LCA (target ion, m/z 372,100%; reference ions, 215, 149% and 257, 51%), DCA (target ion, 255,100%; reference ions, 208, 20% and 370, 13%), CDCA (target ion, 370,100%; reference ions, 255, 31% and 355, 25%), CA (target ion, 253,100%; reference ions, 368, 60% and 458, 35%), HDCA (target ion, 370,100%; reference ions, 255, 72% and 355, 32%), UDCA (target ion, 460,100%; reference ions, 370, 42% and 255, 41%), α-MCA (target ion, 458,100%; reference ions, 443, 25% and 195, 28%), β-MCA (target ion, 195,100%;

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reference ions, 285, 68% and 458, 9%), and ω-MCA (target ion, 195,100%; reference ions, 285, 58% and 369, 29%). Total bile acid concentrations were calculated from summation of individual bile acid concentrations.

Concentrations of Plasma Calcium, Aminotransferases, and Urinary Total Bile Acid. Plasma total calcium levels, alanine and aspartate aminotransferases, urinary total bile acid concentrations, and urinary and plasma creatinine concentrations were measured using Calcium C-Testwako, Transaminase CII-Testwako, Total bile acid-Testwako, and Creatinine Testwako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Urinary bile acid concentrations were normalized with creatinine levels.

Immunoblotting. Kidney membrane preparation was performed as reported previously (Zollner et al., 2006b). Proteins in the membrane fraction were resolved by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA) and probed with a monoclonal antibody against Mrp4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or

an anti-lamin B antibody (Santa Cruz Biotechnology) to confirm the equal protein amount in the lanes. Although Mrp4 has a predicted molecular weight of 150 kDa, it has a number of potential transmembrane regions, glycosylation and phosphorylation sites, which may affect the migration of the protein (Zollner et al., 2006b). Homogenized protein was quantified with a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL).

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction. Total RNAs from samples were prepared by the acid guanidine thiocyanate-phenol/chloroform method and cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega Corporation, Madison, WI) (Ogura et al., 2009). Real-time polymerase chain reaction was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems). Primers for Mrp3 (GenBank accession number NM_029600) were 5'-GCC AAC TTC CTC CGA AAC TA-3' and 5'-CTT GCG GAC CTC GTA GAT GG-3', and others have been reported previously (Ishizawa et al., 2008;

Ogura et al., 2009). Relative mRNA levels were calculated by the comparative threshold cycle method using glyceraldehyde-3-phosphate dehydrogenase as the internal control (Choi et al., 2006).

Statistical Analysis. Data are presented as means ± SD, and statistical differences were determined by ANOVA.

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Results

Modulation of Bile Acid Compositions in Liver and Plasma of Mice fed Bile Acid-Supplemented Chow by 1α(OH)D_3. We fed mice with chow supplemented with 0.4% bile acid for 6 days and examined the effects of bile acid supplements on food intake and liver toxicity in mice. Feeding with CA, DCA and LCA slightly decreased the food intake, but did not induce a significant increase in plasma aminotransferase levels (Table 1). CDCA feeding did not change food intake or plasma aminotransferase levels. Thus, 0.4% bile acid supplements have no or only modest toxic effects in mice. To examine the effects of VDR activation on bile acid metabolism, we fed mice with control chow or chow supplemented with 0.4% bile acid and then administered 1α(OH)D_3 via gavage as shown in Fig.1A. 1α(OH)D_3 is rapidly converted to 1,25$(OH)_2D_3$ after injection and is more effective than 1,25$(OH)_2D_3$ in prolonging survival time of mice inoculated with leukemia cells (Honma et al., 1983). Treatment of mice with 1α(OH)D_3 for 3 days increased plasma calcium levels to 18.5 mg/dL from 10.3 mg/dL of control mice, consistent with effective VDR activation (Table 2). We examined plasma and liver bile acid composition with GC-MS after deconjugation. In mice fed control chow diet,

CA (22%) and ω-MCA (56%) were the major hepatic bile acids (Fig. 2A). CA and β-MCA have been reported to be the major hepatic bile acids in mice (Stedman et al., 2004; Zollner et al., 2006b). Since ω-MCA is a secondary bile acid converted from β-MCA by intestinal bacteria (Eyssen et al., 1983), microflora may influence the concentration of ω-MCA in the bile acid pool. 1α(OH)D_3 treatment decreased the minor bile acid components (LCA, DCA, HDCA, UDCA, CDCA), but did not change total bile acid, CA, or ω-MCA concentrations. CA, HDCA, UDCA and DCA were detected in plasma, and 1α(OH)D_3 decreased plasma total bile acid and DCA concentrations (Fig. 2B).

We examined the effects of 1α(OH)D_3 on bile acid metabolism by feeding mice 0.4% bile acid-supplemented chow as shown in Fig. 1A. First, we fed mice chow supplemented with primary bile acids. CDCA feeding increased the total bile acid concentrations in liver and plasma 2.8-fold and 2.4-fold, respectively, compared with those in mice fed control chow (Figs. 2 and 3). The major bile acid components in the liver of CDCA-fed mice were α-MCA (35%), UDCA (21%), ω-MCA (17%), and CDCA (17%) (Fig. 3A). CDCA is converted to α-MCA and β-MCA in the liver and to UDCA by

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microflora (Botham and Boyd, 1983; Fromm et al., 1983). 1α(OH)D_3 treatment decreased total bile acid concentration, LCA, DCA, HDCA, UDCA, CDCA, β-MCA, and ω-MCA in the liver. CDCA feeding increased plasma CDCA and α-MCA (Fig. 3B). Due to high variation for other bile acids, only the 1α(OH)D_3-dependent decrease in DCA was statistically significant. CA feeding increased liver total bile acids 2.2-fold compared with mice fed control chow and 89% of the liver bile acids were CA (Fig. 4A). Plasma total bile acids were increased in mice fed CA 11-fold compared with mice fed control chow (Figs. 2 and 4), and CA and DCA were the major pool components (Fig. 4B). 1α(OH)D_3 treatment decreased plasma total bile acids and CA, but did not reduce hepatic bile acids.

Next, we examined the effects of 1α(OH)D_3 on mice fed chow supplemented with secondary bile acids. DCA feeding increased total liver bile acids 4.3-fold compared with mice fed control chow (Figs. 2 and 5). The liver of these mice contained CA (72%) and DCA (27%), and 1α(OH)D_3 treatment effectively decreased hepatic total bile acids, DCA and CA (Fig. 5A). Total plasma bile acids were increased 18-fold compared with mice fed control chow, and 1α(OH)D_3 treatment decreased plasma total bile acids (Fig.

5B). Although the effect of 1α(OH)D_3 on plasma DCA was not significant (p = 0.06), the 1α(OH)D_3-induced decrease in the total bile acid concentration in mice fed DCA could be due to enhanced metabolism or elimination of DCA. LCA feeding did not increase the total bile acid concentration of liver or plasma (Figs. 2 and 6). In the liver of mice fed LCA the major bile acids were HDCA (24%) and α-MCA (32%) (Fig. 6A). LCA is metabolized to HDCA by CYP3A enzymes, which are induced by LCA-responsive nuclear receptors, such as PXR and VDR (Xie et al., 2001; Makishima et al., 2002). 1α(OH)D_3 treatment decreased LCA and DCA, but had no effect on total bile acids, HDCA and α-MCA in the liver of LCA-fed mice. 1α(OH)D_3 did not alter plasma bile acid levels (Fig. 6B). Therefore, 1α(OH)D_3 administration enhances the metabolism of bile acids, such as CDCA and DCA.

Bile Acid Metabolism is Enhanced by 1α(OH)D_3 Treatment in Fasted Mice Pre-Fed CDCA-Supplemented Chow. 1α(OH)D_3 treatment induces hypercalcemia and weight loss in mice (Ishizawa et al., 2008). To rule out the possibility that hypercalcemia-associated decreased food intake affects liver and plasma bile acid

concentrations, we fed mice chow supplemented with or without CDCA for 5 days and then administered 1α(OH)D_3 under fasting condition (Fig. 1B). Body weight did not differ between vehicle-treated mice and 1α(OH)D_3-treated mice (Table 3). In mice fed normal chow, 2-day fasting decreased liver bile acid concentrations (Figs. 2 and 7). The major bile acids were CA and ω-MCA, similar to the pool composition in mice fed control chow *ad lib*. Interestingly, the total bile acid concentration in the liver of mice pre-fed CDCA-supplemented chow decreased to control levels after 2 days of fasting, and 1α(OH)D_3 administration further decreased the total hepatic bile acids (Fig. 7A and B). CDCA pre-feeding decreased CA and ω-MCA and increased UDCA, CDCA and α-MCA in the liver, and 1α(OH)D_3 administration decreased bile acids, such as CA and UDCA (Fig. 7B). Although not statistically significant due to high variation, 1α(OH)D_3 likely decreased CDCA and α-MCA. This indicates that the finding of decreased hepatic bile acids by 1α(OH)D_3 is not due to a secondary effect of decreased food intake. 1α(OH)D_3 treatment also likely decreased plasma DCA, CA and UDCA in mice pre-fed CDCA, although these effects were not statistically significant (Fig. 7C). We examined total bile acid concentration in urine and found that 1α(OH)D_3 administration increased urinary

excretion of bile acids, especially in mice pre-fed CDCA-supplemented chow (Fig. 7D). These findings suggest that VDR activation stimulates the excretion of bile acids via urine.

1α(OH)D_3 Treatment Induces Expression of Bile Acid Transporters. We examined mRNA expression of genes involved in bile acid metabolism in mice treated with 1α(OH)D_3. In the liver, bile acids are synthesized from cholesterol by enzymes, such as cholesterol 7α-hydroxylase (Cyp7a1) and sterol 12α-hydroxylase (Cyp8b1), and are catabolized by detoxifying enzymes, such as CYP3A (Xie and Evans, 2001; Russell, 2003). Sodium taurocholate-cotransporting polypeptide (Ntcp) and organic anion transporting polypeptides (Oatps) are involved in bile acid uptake at the basolateral membrane of hepatocytes, and bile acids are excreted by the canalicular bile salt export pump (Bsep) and Mrp2 (Zollner et al., 2006a). At hepatocyte basolateral membrane, Mrp3, Mrp4, and the organic solute transporter α/β (Ostα/β) play a role in alternative expression of bile acids into the system circulation. 1α(OH)D_3 treatment increased liver mRNA expression of Cyp7a1 and Ostα, although it was not effective on expression of

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Cyp24a1, a VDR target gene involved in vitamin D inactivation (Fig. 8A). Expression of enzymes (Cyp8b1 and Cyp3a11) and transporters (Ntcp, Oapt1a1, Oatp1a4, Oatp1b2, Bsep, Mrp2, Mrp3, Mrp4, and Ostβ) was not significantly changed (data not shown). Since VDR does not induce transcription of a target gene in hepatocytes because of low expression of VDR (Gascon-Barre et al., 2003), the effect of $1\alpha(OH)D_3$ treatment on Cyp7a1 and Ostα expression may be through indirect mechanisms. As reported previously (Ishizawa et al., 2008; Ogura et al., 2009), $1\alpha(OH)D_3$ induced mRNA expression of Cyp24a1 in the kidney and small intestine (Fig. 8B and C), indicating that $1\alpha(OH)D_3$ treatment effectively activates VDR in these tissues. The bile acid transporters Mrp2, Mrp4, and Ostα/β are expressed in renal tubular cells and are thought to be involved in urinary bile acid excretion (Zollner et al., 2006a). $1\alpha(OH)D_3$ increased renal mRNA expression of Mrp2, Mrp3, and Mrp4 (Fig. 8B), but not Ostα or Ostβ (data not shown). Asbt, Mrp3, and Ostα/β are suggested to be involved in bile acid transport in enterocytes (Zollner et al., 2006a). Treatment of mice with $1\alpha(OH)D_3$ increased intestinal mRNA expression of Asbt and Mrp4 (Fig. 8C), but not Mrp2, Mrp3, Ostα, or Ostβ (data not shown).

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Since 1α(OH)D_3 administration increased urinary excretion of bile acids (Fig. 7D) and renal mRNA expression of bile acid transporters (Fig. 8B), we examined protein expression of renal bile acid transporters. Immunoblotting analysis showed increased renal expression of Mrp4 protein in mice treated with 1α(OH)D_3 (Fig. 8D). These findings indicate that VDR activation stimulates bile acid excretion through increased bile acid transporter expression in kidney.

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Discussion

In this study, we report that 1α(OH)D_3 treatment enhances bile acid metabolism *in vivo*. A bile acid-supplemented diet increased liver bile acid concentrations, indicating that bile acids absorbed from the intestine accumulate in the liver. In the liver of mice fed CDCA, DCA, and LCA, the major bile acids detected in the liver were α-MCA (Fig. 3A), CA (Fig. 5A), and α-MCA (Fig. 6A), respectively. Alternatively, CA was the major bile acid in the liver of mice fed CA (Fig. 4A). These findings indicate that supplemented CDCA, DCA and LCA are metabolized effectively but CA is resistant to metabolism. CA and β-MCA have been reported be major hepatic bile acids in mice (Stedman et al., 2004; Zollner et al., 2006b), and α-MCA is thought to be a precursor of β-MCA (Cherayil et al., 1963) (Fig. 9). Hepatic bile acids from the enterohepatic circulation may be subjected to conversion to primary bile acids, CA and α-MCA, although a detailed mechanism of bile acid metabolism in rodents remains to be elucidated. 1α(OH)D_3 treatment decreased several bile acid components in the liver of mice fed normal chow (Fig. 2A), and this effect was observed more clearly in mice fed CDCA (Fig. 3A). Similar effects of 1α(OH)D_3 on hepatic bile acid compositions were

observed in mice administered 1α(OH)D_3 under fasting condition (Fig. 7B). Compared to the decrease in hepatic bile acids, such as CDCA, UDCA and ω-MCA, in 1α(OH)D_3-treated mice, we did not detect increased bile acid components. These findings suggest that 1α(OH)D_3 treatment stimulates bile acid metabolism, particularly enhancing transport for excretion. Bile acids are excreted from hepatocytes into bile ducts by Bsep and Mrp2 (Zollner et al., 2006a) (Fig. 9). At the hepatocyte basolateral membrane, Mrp3, Mrp4, and Ostα/β play a role in the alternative excretion of bile acids into the systemic circulation. 1α(OH)D_3 treatment increased mRNA expression of Cyp7a1 and Ostα in the liver (Fig. 8A). Cyp7a1 catalyzes the rate-limiting step of the classical bile acid synthesis pathway and is negatively regulated by the bile acid receptor FXR (Makishima, 2005). The induction of Cyp7a1 may be due to a decrease in FXR-activating bile acids by excretion from hepatocytes. The bile acid transporters Mrp2, Mrp4, and Ostα/β are thought to be involved in urinary bile acid excretion (Zollner et al., 2006a; Alrefai and Gill, 2007). Although the role of Mrp3 in the renal bile acid transport has not been elucidated, Mrp3 is a direct target gene of VDR (McCarthy et al., 2005). 1α(OH)D_3 treatment increased mRNA expression of kidney Mrp2, Mrp3, and Mrp4 (Fig.

8B). We also observed increased protein expression of kidney Mrp4 (Fig. 8D) and increased urinary excretion of bile acids in mice treated with 1α(OH)D_3 (Fig. 7D). These findings suggest that 1α(OH)D_3 treatment decreases hepatic bile acids by inducing bile acid transporters for urinary excretion (Fig. 9).

In DCA-fed mice, 1α(OH)D_3 treatment decreased hepatic DCA effectively but was less effective in altering CA concentration (Fig. 5A). 1α(OH)D_3 treatment was not effective in reducing accumulated CA in the liver of mice fed CA (Fig. 4A). VDR-induced mechanisms may be ineffective in elimination of accumulated CA in the liver. In contrast to hepatic CA, the plasma CA concentration was decreased after 1α(OH)D_3 treatment in mice fed CA (Fig. 4B). Plasma bile acid levels were also increased in mice fed DCA (Fig. 5B), but not in mice fed CDCA (Fig. 3B) or LCA (Fig. 6B). Increased plasma CA and DCA may be regulated by VDR-induced excretion into urine. Although LCA is an endogenous ligand for VDR (Makishima et al., 2002), 1α(OH)D_3 administration was not effective in decreasing bile acid concentrations in mice fed LCA (Fig. 6A). Although hepatic LCA and DCA were decreased by 1α(OH)D_3 treatment, accumulated HDCA and α-MCA were not affected. HDCA and α-MCA may

be products of LCA metabolism by LCA-activated VDR and VDR-induced mechanisms may not be effective in the further elimination of HDCA and α-MCA.

Bile acids are essential detergents that are required for the ingestion and intestinal absorption of hydrophobic nutrients, including vitamin D (Hofmann, 1999). VDR has dual functions as an endocrine receptor for $1,25(OH)_2D_3$ and as a metabolic sensor for secondary bile acids, such as lithocholic acid (Makishima et al., 2002). Although several genes involved in bile acid metabolism, such as CYP3A4 and MRP3, have been reported to be VDR target genes (McCarthy et al., 2005; Matsubara et al., 2008), the *in vivo* role of VDR as a bile acid sensor may be limited. While administration of high concentrations of LCA restored serum calcium levels to the normal range in vitamin D-deficient rats by increasing VDR target gene expression and bone calcium mobilization, it was not effective in rats with normal vitamin D levels (Nehring et al., 2007). These findings indicate that LCA can substitute for vitamin D in calcium homeostasis only in vitamin D-deficient rats. We demonstrated in this study that pharmacological doses of vitamin D enhance bile acid metabolism. $1,25(OH)_2D_3$ increases plasma bile clearance of vitamin D_3 and $1,25(OH)_2D_3$ (Gascon-Barre and

Gamache, 1991). While hepatocytes express low levels of VDR, biliary epithelial cells express functional VDR (Gascon-Barre et al., 2003). VDR activation in biliary epithelial cells may influence biliary excretion of bile acids as well as vitamin D compounds. In addition, the extracellular calcium-sensing receptor is expressed in hepatocytes and its activation enhances bile flow (Canaff et al., 2001). Increased blood calcium by vitamin D administration may also influence bile acid elimination. $1\alpha(OH)D_3$ administration is not effective in altering bile acids accumulated in bile duct-ligated mice (Ogura et al., 2009). This may be due to artificial obstruction of bile flow. About 20 enzymes have been shown to be involved in bile acid synthesis in the liver (Russell, 2003). The mechanisms of catabolism and transport of bile acids in the liver and kidney remain to be elucidated. The investigation of vitamin D-regulated bile acid metabolism will be helpful for understanding bile acid metabolism, especially elimination, and in the prevention and treatment of bile acid-associated diseases.

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Footnote

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Figure legends

Fig. 1. Experimental procedure for bile acid feeding and 1α(OH)D_3 treatment in mice. (A) Mice were fed standard or 0.4% bile acid-supplemented chow for 8 days. Mice were administrated 1α(OH)D_3 via gavage at days 6, 7, and 8, and sacrificed at day 9. (B) Mice were fed standard or 0.4% CDCA-supplemented chow for the first 5 days and were then fasted from day 6. 1α(OH)D_3 was administered to fasted mice on day 6 and 7. Mice were sacrificed for sample collection at day 8.

Fig. 2. Bile acid compositions in liver (A) and plasma (B) in mice fed standard chow. Mice were fed standard chow and administered 1α(OH)D_3 as shown in Fig.1A. *, $p < 0.05$ compared with vehicle control to 1α(OH)D_3 administration. ND, not detected.

Fig. 3. Bile acid compositions in liver (A) and plasma (B) in mice fed CDCA-supplemented chow. Mice were fed 0.4% CDCA-supplemented chow and administered 1α(OH)D_3 as shown in Fig.1A. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle control to 1α(OH)D_3 administration.

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Fig. 4. Bile acid compositions in liver (A) and plasma (B) in mice fed CA-supplemented chow. Mice were fed 0.4% CA-supplemented chow and administered 1α(OH)D_3 as shown in Fig.1A. *, $p < 0.05$; **, $p < 0.01$ compared with vehicle control to 1α(OH)D_3 administration. ND, not detected.

Fig. 5. Bile acid compositions in liver (A) and plasma (B) in mice fed DCA-supplemented chow. Mice were fed 0.4% DCA-supplemented chow and administered 1α(OH)D_3 as shown in Fig.1A. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle control to 1α(OH)D_3 administration. ND, not detected.

Fig. 6. Bile acid compositions in liver (A) and plasma (B) in mice fed LCA-supplemented chow. Mice were fed 0.4% LCA-supplemented chow and administered 1α(OH)D_3 as shown in Fig.1A. *, $p < 0.05$ compared with vehicle control to 1α(OH)D_3 administration. ND, not detected.

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Fig.7. Effects of 1α(OH)D_3 on bile acid composition in mice pre-fed CDCA-supplemented chow. Bile acid compositions in the liver of mice fed standard chow (A) and 0.4% CDCA-supplemented chow (B). (C) Bile acid compositions in plasma in mice fed 0.4 % CDCA-supplemented chow. (D) Total bile acid concentrations in urine. Mice were fasted after feeding and administered 1α(OH)D_3 as shown in Fig. 1B. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle control to 1α(OH)D_3 administration. ND, not detected.

Fig. 8. Effects of 1α(OH)D_3 on mRNA expression of genes involved in bile acid metabolism in liver (A), kidney (B), and intestine (C), and on renal Mrp4 protein expression (D). Total RNA was prepared from the liver, kidney, and small intestine of mice fed standard chow with or without 1α(OH)D_3 treatment as shown in Fig. 1A (n=6/each), and expression of the indicated genes was measured with real-time quantitative polymerase chain reaction using glyceraldehyde-3-phosphate dehydrogenase as the internal control. Values for normalized mRNA expression are relative to those of vehicle control-treated mice. The values represent the means ± SD. n.s., not significant; *,

$p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Proteins from the kidney were subjected to immunoblotting. Each lane was loaded with 41 μg of membrane proteins. Experiments were repeated with different mouse samples with similar results.

Fig. 9. Model of effects of VDR ligand on bile acid metabolism. Primary bile acids, CA and CDCA, are generated from cholesterol by liver enzymes, including Cyp7a1 and Cyp8b1, and are secreted in bile as glycine or taurine conjugates. CDCA is metabolized to α-MCA and β-MCA in the rodent liver. Bsep and Mrp2 are localized in the canalicular membrane of hepatocytes and transport bile acids into bile. Most bile acids are reabsorbed in the intestine and recirculate to the liver through the portal vein in a mechanism called the enterohepatic circulation. Bile acids that escape reabsorption are converted to the secondary bile acids DCA, LCA, UDCA, and ω-MCA by intestinal microflora. Portions of the secondary bile acids enter the enterohepatic circulation from the colon. The transporters Asbt, Mrp3, Ostα/β are involved in bile acid absorption in the intestine. At the basolateral membrane of hepatocytes, Ntcp and Oatps uptake bile acids from the portal circulation. The basolateral transporters Mrp3, Mrp4, and Ostα/β play a

role in the alternative excretion of bile acids from hepatocytes into the systemic circulation. Renal Mrp2, Mrp4, Ostα/β are thought to be involved in urinary bile acid excretion. VDR activation induces expression of bile acid transporters, such as liver Ostα, kidney Mrp2, and Mrp4, and stimulates urinary excretion of bile acids. Expression of liver Cyp7a1, intestinal Asbt, Mrp4, and renal Mrp3 are also induced by VDR activation. Thus, VDR, which responds to both $1,25(OH)_2D_3$ and LCA, plays a role in regulation of bile acid metabolism.

TABLE 1

Cumulative food intake and plasma transaminase levels in mice fed bile acid-supplemented chow

Mice were fed 0.4% bile acid-supplemented chow for 6 days (n=5 for each condition). Values represent mean ± SD.

Bile acid in diet	Cumulative food intake (g)	Plasma ALT (IU/l)	Plasma AST (IU/l)
Control	16.7 ± 0.5	10.8 ± 12.3	42.5 ± 14.7
CDCA	16.0 ± 0.5	18.0 ± 13.6	79.9 ± 31.9
CA	12.2 ± 0.9	23.1 ± 5.2	101.5 ± 14.8
	$p < 0.001$[a]		
DCA	13.3 ± 0.9	26.3 ± 9.4	115.7 ± 50.8
	$p < 0.001$[a]		
LCA	14.5 ± 1.4	11.5 ± 2.0	84.6 ± 16.7
	$p < 0.001$[a]		

[a]Comparison with control chow.

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

TABLE 2

Body weight and plasma calcium levels in mice fed bile acid-supplemented chow and treated with $1\alpha(OH)D_3$

Mice were fed 0.4% bile acid-supplemented chow and treated with vehicle (- $1\alpha(OH)D_3$) or $1\alpha(OH)D_3$ (+ $1\alpha(OH)D_3$) as shown in Fig.1A. Values for body weight and plasma calcium levels represent mean ± SD.

Bile acid in diet	Body weight (g)		Plasma calcium (mg/dL)		Number of mice	
	- $1\alpha(OH)D_3$	+ $1\alpha(OH)D_3$	- $1\alpha(OH)D_3$	+ $1\alpha(OH)D_3$	- $1\alpha(OH)D_3$	+ $1\alpha(OH)D_3$
Control	20.1 ± 1.6	15.2 ± 1.3 $p < 0.001$[a]	10.3 ± 1.7	18.5 ± 2.1 $p < 0.001$[a]	5	7
CDCA	22.1 ± 2.4	20.0 ± 1.8	11.9 ± 0.7	15.0 ± 1.6 $p < 0.001$[a]	8	8
CA	22.1 ± 1.7	19.2 ± 1.4 $p < 0.01$[a]	6.6 ± 1.1	11.6 ± 1.2 $p < 0.001$[a]	8	8
DCA	20.1 ± 0.7	14.9 ± 1.1 $p < 0.001$[a]	9.4 ± 2.8	12.8 ± 1.7 $p < 0.05$[a]	8	8
LCA	22.4 ± 1.3	19.5 ± 0.8 $p < 0.001$[a]	9.7 ± 1.3	15.2 ± 4.4 $p < 0.001$[a]	8	7

[a]Comparison with vehicle control without $1\alpha(OH)D_3$ treatment.

TABLE 3

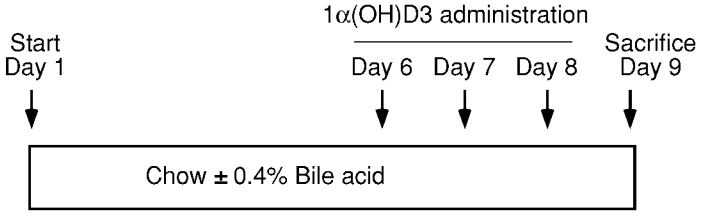
Body weight and plasma calcium levels in mice pre-fed bile acid-supplemented chow and treated with 1α(OH)D_3 under fasting

Mice were pre-fed 0.4% CDCA-supplemented chow and treated with vehicle (- 1α(OH)D_3) or 1α(OH)D_3 (+ 1α(OH)D_3) under fasting conditions as shown in Fig.1B. Values for body weight and plasma calcium levels represent mean ± SD.

Bile acid in diet	Body weight (g)		Plasma calcium (mg/dL)		Number of mice	
	- 1α(OH)D_3	+ 1α(OH)D_3	- 1α(OH)D_3	+ 1α(OH)D_3	- 1α(OH)D_3	+ 1α(OH)D_3
Control	20.5 ± 0.5	20.2 ± 1.3	6.0 ± 0.4	6.8 ± 0.6	5	7
				$p < 0.05$[a]		
CDCA	20.7 ± 1.5	20.5 ± 0.8	7.1 ± 0.7	7.6 ± 1.5	6	6

[a]Comparison with vehicle control without 1α(OH)D_3 treatment.

A


1α(OH)D3 administration
Start
Day 1
Day 6
Day 7
Day 8
Sacrifice
Day 9
Chow ± 0.4% Bile acid

B

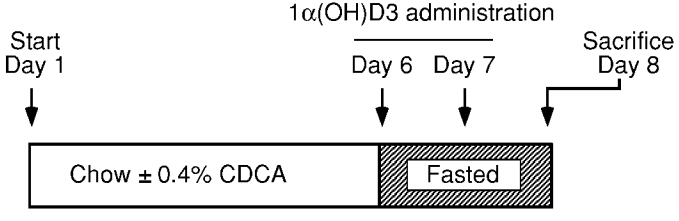
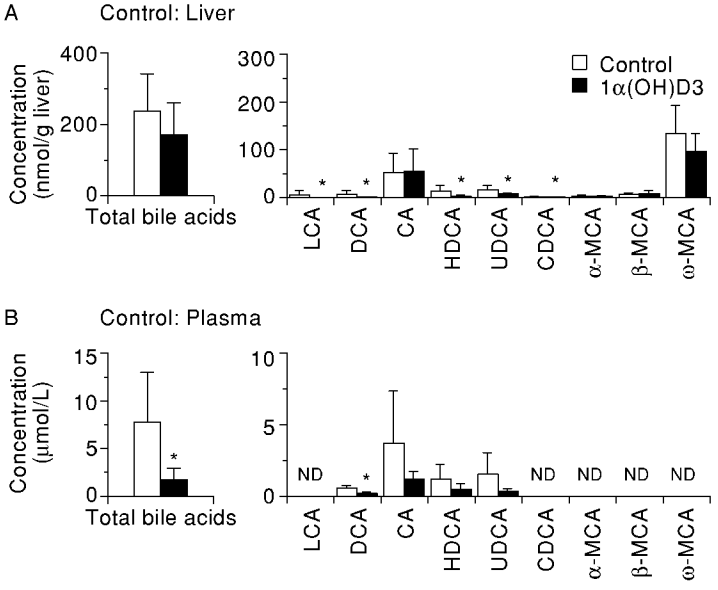

1α(OH)D3 administration
Start
Day 1
Day 6
Day 7
Sacrifice
Day 8
Chow ± 0.4% CDCA
Fasted

Fig. 1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200
100
0
Control
1α(OH)D3
*
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA
B
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Concentration (μmol/L)
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5
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Total bile acids
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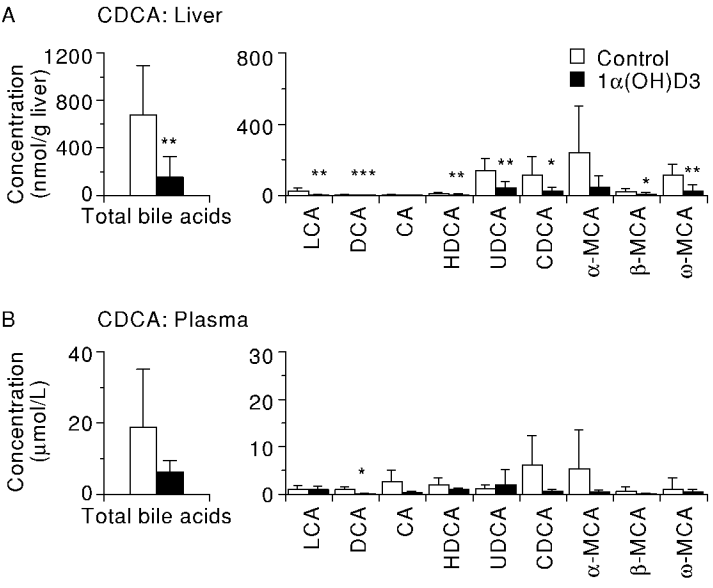
5
0
ND
*

Fig. 2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CDCA: Liver
Concentration (nmol/g liver)
1200
800
400
0
**
Total bile acids
800
400
0
Control
1α(OH)D3
**
***
**
**
*
*
**
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA
B
CDCA: Plasma
Concentration (μmol/L)
40
20
0
Total bile acids
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20
10
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*
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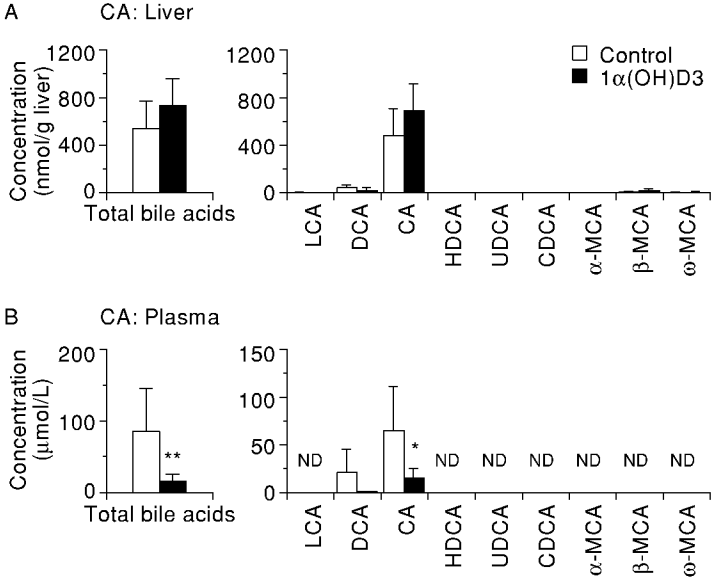
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA

Fig. 3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Control
1α(OH)D3
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA
B
CA: Plasma
Concentration (μmol/L)
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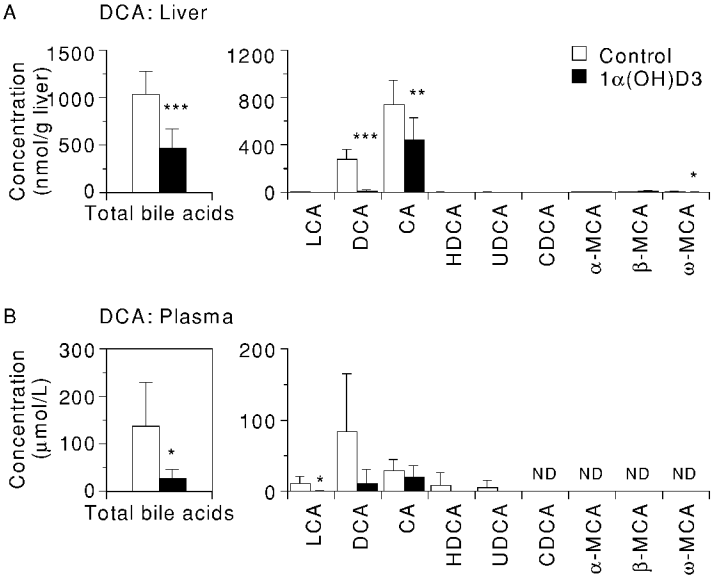
100
0
150
50
**
*
ND

Fig. 4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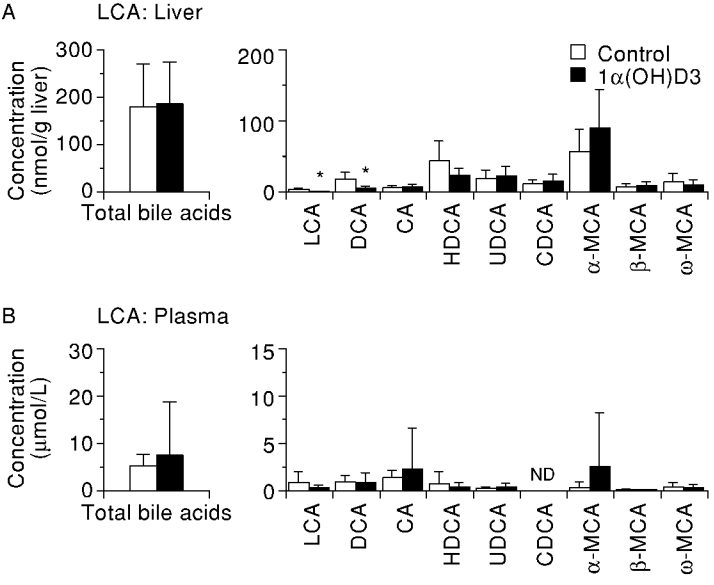
1000
500
0
***
Total bile acids
1200
800
400
0
***
**
*
Control
1α(OH)D3
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA
B
DCA: Plasma
Concentration (μmol/L)
300
200
100
0
*
Total bile acids
200
100
0
*
ND
ND
ND
ND
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA

Fig. 5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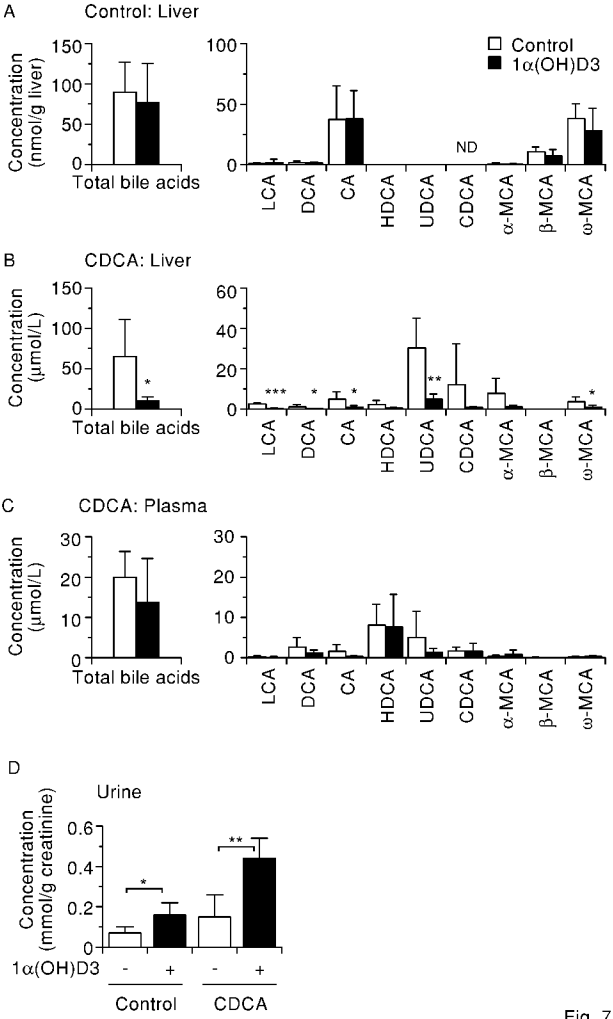
LCA: Liver
Concentration (nmol/g liver)
300
200
100
0
Total bile acids
200
100
0
Control
1α(OH)D3
*
*
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA
B
LCA: Plasma
Concentration (μmol/L)
30
20
10
0
Total bile acids
15
10
5
0
ND
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA

Fig. 6


A
Control: Liver
Concentration (nmol/g liver)
Total bile acids
Control
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ND
LCA
DCA
CA
HDCA
UDCA
CDCA
α-MCA
β-MCA
ω-MCA
B
CDCA: Liver
Concentration (μmol/L)
Total bile acids
C
CDCA: Plasma
Concentration (μmol/L)
Total bile acids
D
Urine
Concentration (mmol/g creatinine)
1α(OH)D3
Control
CDCA

Fig. 7

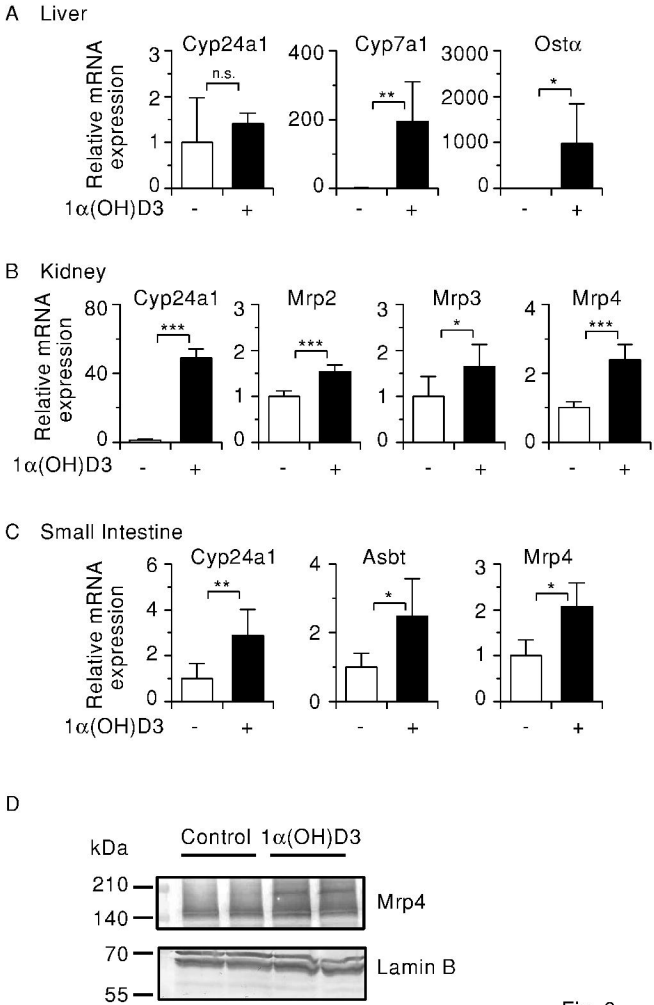
A Liver
Relative mRNA expression
Cyp24a1
n.s.
Cyp7a1
**
Ostα
*
1α(OH)D3 - +
B Kidney
Cyp24a1
***
Mrp2
***
Mrp3
*
Mrp4
***
C Small Intestine
Cyp24a1
**
Asbt
*
Mrp4
*
D
kDa
Control
1α(OH)D3
210
140
Mrp4
70
55
Lamin B

Fig. 8

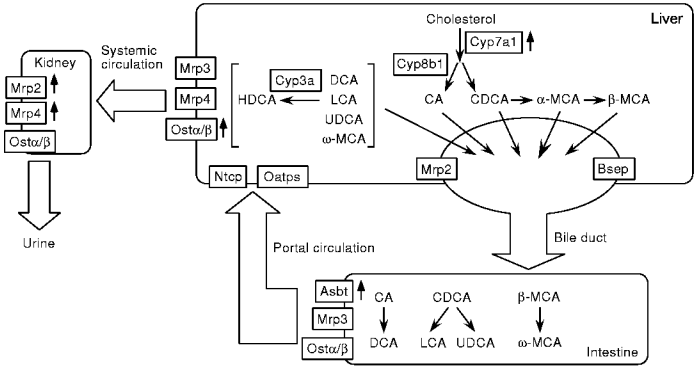
Liver
Cholesterol
Cyp7a1
Cyp8b1
CA
CDCA
α-MCA
β-MCA
Cyp3a
DCA
HDCA
LCA
UDCA
ω-MCA
Kidney
Mrp2
Mrp4
Ostα/β
Urine
Systemic circulation
Mrp3
Mrp4
Ostα/β
Ntcp
Oatps
Mrp2
Bsep
Bile duct
Portal circulation
Asbt
Mrp3
Ostα/β
CA
CDCA
β-MCA
DCA
LCA
UDCA
ω-MCA
Intestine

Fig.9